INVOLVEMENT OF ACETYLSALICILYC ACID IN SUNFLOWER (*HELIANTHUS SP.*) PLANT RESPONSE TO DIFFERENT ABIOTIC AND BIOTIC STRESS

Cornelia Purcărea, Adriana Chiş, Nicolae Csep
University of Oradea
Faculty for Environmental Protection , 26 General Magheru str., Oradea,
email: neli_oradea@yahoo.com

ABSTRACT

In our researches we studied the influence of exogenous acetylsalicylic acid (ASA) in the plant response to abiotic and biotic stress. Salt stress, an abiotic stress, determine modification of some biochemical indicators, like, antioxidant enzymes, proline (amino acid accumulate in higher plants under salinity stress) content, assimilatory pigment content. Pre-treatment of sunflower seeds with ASA may cause a low level of oxidative stress, improving the antioxidative capacity of the plants. Acetylsalicylic acid can increase the plant tolerance to salt stress induced in our experiment by 150 mM NaCl treatments. Moreover we studied the role of ASA in sunflower resistance, its antifungal effect against fungal pathogens (*Botrytis cinerea* and *Sclerotinia sclerotiorum*), which has significant influence on the level and quality of the production of this plant.

1. INTRODUCTION

Acetylsalicylic acid (ASA), and salicylic acid (SA) play act as a potential non-enzymatic antioxidant as well as a plant growth regulator. Plant growth and development was intensly affected by different adverse environmental conditions and by pathogens. In plants the damaging effects of these abiotic and biotic stress factors take the shape of alterations in the plant physiology which leads to a reduction of growth and a decrease of their bioproductivity. Salicylic and acetylsalicylic acid could ameliorate the damaging effects of heavy metals in rice (Mishra and Choudhuri, 1999), drought stress in wheat (Waseem et al, 2006) and salt stress in wheat (Arfan et al, 2007) and in sunflower plants (Noreen et al, 2009).

Gutiérrez-Coronado et al 1998, found that SA sprayed on leaves increases significantly the root growth in soybean plants, and Gutiérrez-Rodríguez et al 1991, found that SA stimulated root growth in carrot, radish, and beet plants. Its important to know if SA stimulated root growth in ligneous species such as *Pinus patula* Schl. Et Cham, one specie extensively planted in parks, gardens and forests of México (Perry, 1991).

However the leaves of corn and soybean treated with acetylsalicylic acid or gentisic acid exhibited no change in their chlorophyll contents (Khan et al, 2003). Salicylic acid activated the synthesis of carotenoids, xanthophylls and the rate of de-epoxidation but decreased the level of chlorophyll pigments, both in wheat and moong plants also the ratio of chlorophyll a/b, in wheat plantlets (Moharekar et al., 2003).
Acetylsalicylic acid used in optimal concentrations can temporarily reduce the oxidative stress level in plants improving their antioxidative capacity and stimulating the synthesis of some protective components like proline.
Salicylic and acetylsalicylic acid are important signaling (signalling) molecules involved in plant defense in both locally and systemically induced disease resistance responses.

This substances are largely used compounds in human and veterinary farmacopee (MedEx 2009, Memomed 2009). They are involved in the installation of Systemic Acquired Resistance (SAR), and play an active role in plant defense on viral, fungal and bacterian pathogens (Csep and Sesan, 1996, Antofie et al.2003).

Loake and Grant (2007) relieved that SA is synthesised by plants in response to challenge by a diverse range of phytopathogens and is essential to the establishment of both local and systemic-acquired resistance (SAR). Salicylic acid application induces accumulation of pathogenesis-related (PR) proteins.

Recent advances in our understanding of plant defence signalling have revealed that plants employ a network of signal transduction pathways, some of which are independent of salicylic acid. Cross-talk between the salicylic acid-dependent and the salicylic acid-independent pathways provides great regulatory potential for activating multiple resistance mechanisms in varying combinations (Pieterse and van Loon, 1999).

2. MATHERIALS AND METHODS

2.1.Sample preparation

Surface sterilized sunflower seeds (*Helianthus sp.* L) were soaked for 12 h in water or in 0.1 mM ASA.

The germination was made in plastic recipients, 7 days, on a filter paper, moistened with 20 ml treatment solution:

- Control lot (C) – 12 h soaked in water and germinated in water.
- Sample 1 (S_1) – 12 h soaked in water and germinated in 150 mM NaCl solution;
- Sample 2 (S_2) – 12 h soaked in 0.1 mM ASA and germinated in 150 mM NaCl solution.

Each recipient contained 20 seeds. The germination was made on filter paper moistened with tape water, at 20±3 °C in a Sanyo MLR 351H phytotron, day/night, and relative humidity 65-85%, under natural photon flux density. Every day, the quantity of solutions from the recipients was brought to the level of 20 ml.

After 7 days of germination we planted the plantlets in sand, leaving them there for an additional 7 days, and sprayed their primary leaves each day with 1 ml of 0.1 mM ASA solutions or with water for the control lot.

The experiments were performed in the Agrifood Biochemistry laboratory of Faculty for Environmental Protection Oradea.

2.2. Preparation of enzme extract

0,5g fresh sample (roots and leaves) were collected from each variant in the 14-th day of germination, and were blended with 8 ml phosphate buffer solution, pH 7.0, diluted 1:9 with distilled water, cooled at 4°C. The samples were centrifuged at 15000 x g, for 20 minutes at 4°C, and the supernatant was separated. The extract is kept in the refrigerator, for 2 hours for stabilizing and expressing enzyme activity.

2.3. Peroxidase activity determination

Peroxidase activity (POX) was determined at 30°C, with a Shimadzu–UV-mini–1240 spectrophotometer, following the formation of tetraguaiacol at 470 nm wavelength, ε=26.6mM^{-1}cm^{-1}, in a 3 ml reaction mixture containing 1 ml of 0.1 M phosphate buffer, pH=6.0, 1 ml 15mM guaiacol, 1 ml of 3 mM H_2O_2, and 50µl of enzyme extract. One unit of peroxidase activity (U) represents the amount of enzyme catalyzing the oxidation of 1 µmole of guaiacol in 1 min, method cited by Kim and Yoo, 1996.

2.4. Catalase (CAT) activity determination

The decomposition of hydrogen peroxide is followed at 240 nm spectrophotometrically (Aeby, 1984). For preparation of the plant extract 0.5 g plant material was homogenizing with 3 ml 0.1 M Na-phosphate buffer (pH = 6.5).

We measure into a quarz cuvette: 2 ml above phosphate buffer (pH = 6.5), 100 µl hydrogen peroxide solution (12.5 mM concentration), 50 µl plant extract. The solution is mixed, then the absorption change is registered for 3 min at 240 nm. Slope value (reaction velocity: Δ Extinction per Δ time) is calculated.

Then:

$$\text{activity} = \frac{2.15\ \text{ml}}{0.05\ \text{ml}} \cdot \frac{1}{0.04\ \text{mM}^{-1}} \cdot \frac{\Delta E}{\Delta t\ (\text{min})} \cdot \frac{3\ \text{ml}}{0.5\ \text{g}} = 6450 \frac{\Delta E}{\Delta t}$$

Where 0.040 mM^{-1}cm^{-1} is the extinction coefficient of H_2O_2 at 240 nm
Unit: µmol hydrogen peroxide g FW^{-1} min^{-1}

2.5. Proline determination

Proline was determined following Bates et al (1973). For the proline determination 0.5 g of plant material was homogenized 10 ml of 3% aqueous sulfosalicylic acid and the homogenate filtered throught Whatman Nr.2 filter paper. 2 ml of filtrate was treated with 2 ml acid ninhydrin and 2 ml glacial acetic acid in a test tube for 1 hour at 100°C, and the reaction terminated in an ice bath. The reaction mixture was extracted with 4 ml toluene, mixed vigorously with a test tube stirrer for 15-20sec. The cromophore containing toluene was aspired from the aqueous phase warmed to room temperature and the absorbance read at 520 nm using toluene for a blank. The proline concentration was determined from a standard curve and calculated on a fresh weight basis as follows:

[µg proline/ml x ml toluene / 115.5 µg/ µmole]/ [g sample/5] = µmoles proline/g of fresh weight material.

2.6. Assimilatory pigments

After 14th day we determined the content of chlorophyllian pigments of the sunflower plantlets primary leaves, using *N,N-dimethylformamide,* 99.9%, (Moran and Porath, 1980) for the extraction. The extraction of assimilatory pigments in higher plant tissue using *N,N-dimethylformamide* (DMF), expedites the process and enables the determination of small samples with low pigment level (Moran, 1982). There is a vast array of solvents used for the extraction and determination of the chlorophyllian pigments, but most of them necessitate grinding and centrifuging of material with or

without heating. The use of DMF renders the process simpler and faster, since the pigments can be extracted from intact tissue. For extraction, 50 mg fresh weight of primary leaves, were collected separately from each sample, and were blended with 5ml DMF and then cooled at 4°C for 72 hours. The supernatant was separated and the content of the pigment was determined using a UV-visible mini-1240 Shimadzu spectrophotometer, at 664 nm wave length for chlorophyll a, 647 nm for chlorophyll b and 480 nm for carotenoids.
The data obtained after the spectrophotometrycal determination, was mathematically processed using formulae proposed by Moran and Porath (1980).

Chlorophyll a (mg/g sp) = $(11.65\ a_{664} - 2.69\ a_{647}) \cdot V/sp$
Chlorophyll b (mg/g sp) = $(20.81\ a_{647} - 4.53\ a_{664}) \cdot V/sp$
Carotenoids (mg/g sp) = $(1000\ A_{480} - 1.28$ chloroph.a $- 56.7$ chloroph.b$)/\ 245 \cdot V/sp$

The results obtained for all parameters are averages of 3 determinations and were statistically processed by the "t- test" using *Prisma 5 for Windows*. The values of the probabilities were determined from tables using the values of the "t" distribution and the freedom degrees based on which the variance of the empiric series was calculated.

2.7. Antifungal effect of ASA

Biological activity of ASA was studied in preliminary tests made in vitro and in vivo conditions. These tests were performed at the Agricultural Research Station Oradea. The performed tests shows a promising antifungal effect of ASA produced by Sinteza Chemical Work Oradea. ASA was tested as crystals and powder concerning their effect in vitro on the growths dimension of two parasitic fungi of sunflower, *Botrytis cinerea* and *Sclerotinia sclerotiorum*. We used local isolates of these very important parasitic fungi of the sunflower and another plants in the Western part of Romania. In the field testing we used our autohton hybrids (Fundulea 90, Felix), in natural and artificial inoculation with these pathogens.

3. RESULTS AND DISCUSSION

Peroxidase (POX) activity significantly increased ($p<0.005$) in roots of sunflower seedling and a very significant increased ($p<0.001$) was registered in leaves of sunflower after treatment with 150 mM NaCl solution, in the 14-th day of experiment, in comparison with the control lot, germinated in tape water. The soaking and the foliar treatment with 0.1 mM acetylsalcylic acid (ASA) has a protective effect during salt stress. In leaves of sunflower seedling POX activity decreased very significantly, but remained almost unchanged in the roots of seedlings in comparison with salt treated seedling (tabel 1, fig. 1).
Studying the CAT activity in the roots and leaves of sunflower seedlings, we observed a very significant increase of this activity in stressed seedling in comparison with the control lot (unstressed). The CAT activity decreased very significant in ASA treated seedling in comparison with the same parameters in stressed seedling (table1, fig.1).

Table 1. Estimative mean values for the antioxidant enzymes activity seedling in roots and leaves of sunflower seedlings after 14 days of experiment.

		Antioxidant enzymes	
		Peroxidase (POX) (U)	**Catalase (CAT) (U)**
Control lot	**root**	0.013± 0.0026	2.43±0.0264
	leaf	0.01±0.0017	2.86±0.0655
150 mM Salt treatment	**root**	0.02±0.0101 *	9.52±0.3377 ***
	leaf	0.087±0.0055 ***	11.43±0.1587 ***
0.1mM ASA and 150 mM Salt treatment	**root**	0.018±0.0052 *	3.33±0.204 ***
	leaf	0.032±0.0026 ***	6.19±0.0916 ***

p>0.05= not significant; p<0.05 * significant; p<0.01=** distinctly significant; p<0.001=*** very significant in comparison with control lot.

The antioxidant enzymes activity like peroxidase (POX) and catalase (CAT), enhanced by salt treatment. The highest value for POX activity was registred in leaves of sunflower seedling. In case of CAT activity the values of the enhancements of the enzyme extracts from both, roots and leaves, were similar. Bandeoglu et al, 2004, observed that upon salt stress no significant enhancement in activity of antioxidant enzymes was registered, in roots of lentil, but a higher activity was present when compared with the leaf tissue. These results suggested that roots tissue of lentil is protected better from salt stress induced oxidative damage. Acetylsalicylic acid pre-treatment ameliorate the peroxidase and catalase activity under salt stress in enzymes extracts of sunflower seedlings.

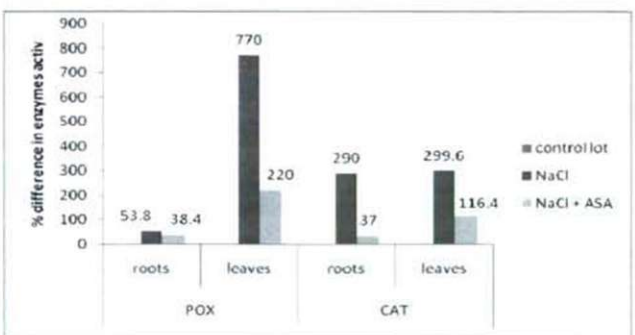

Fig.1. Percentage differences of the activity of antioxidant enzymes, POX and CAT, measured in roots and leaves of sunflower seedlings, in comparison with the same parameters measured in the leaves of sunflower plantlets from the control lot sprayed with water. The value for the control lot was considered 100% (marked with 0 on the chart).

Studying the content of chlorophyllian pigment (chlorophyll a and b) and carotenoids on the leaves of the sunflower seedling obtained from each experimental variant, we observed that the content of assimilatory pigments decreased very significantly after salt stress, but the pre-soaking and the foliar treatment with the 0.01 mM ASA solution,

reduced this difference, in comparison with the control lot (table 2 and fig 2). Similar results were obtained by Kaydan et al. 2007, they observed that under the influence of salinity the photosynthetic pigments greatly decreased. El Tayeb in 2005 found that chl a, b and carotenoids decreased significantly in NaCl treated plants in comparison to controls of barley plants.

Table 2. Estimative mean values for the assimilatory pigments content of the sunflower seedling leaves after treatment salt stress or with ASA solutions treatment.

	Assimilatory pigments		
	chl a (mg/g)	**chl b (mg/g)**	**caroten (mg/g)**
Control lot	1.247±0.0151	0.623±0.0132	0.322±0.006
150 mM Salt treatment	1.158±0.005 ***	0.376±0.0078 ***	0.245±0.0096 ***
0.1mM ASA and 150 mM Salt treatment	1.200±0.006 **	0.445±0.0096 ***	0.308±0.0051 *

p>0.05= not significant; p<0.05 * significant; p<0.01=** distinctly significant; p<0.001=*** very significant in comparison with control lot.

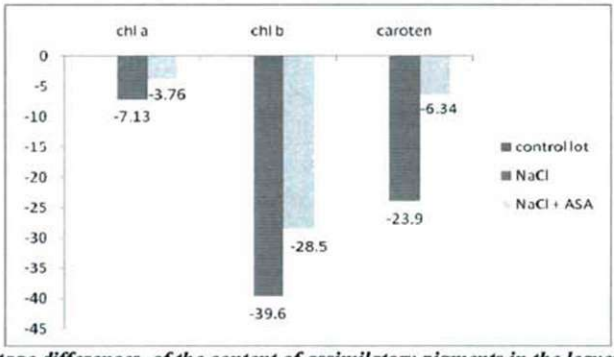

Fig.2. Percentage differences of the content of assimilatory pigments in the leaves of sunflower seedlings, in comparison with the same parameter measured in the leaves of sunflower plantlets from the control lot sprayed with water. The value for the control lot was considered 100% (marked with 0 on the chart).

Under stress condition, free proline (amino acid accumulate in higher plants under salinity stress) level increased in the leaves of sunflower seedlings. Studying the value after spectrophotometrycal determination of proline content, we observed that under salt stress the proline content increased very significantly, with 75,9%, in comparison with control lot. The treatment with 0.1mM ASA alleviated the effect of salt stress, and the free proline content in this condition was lower (with 29%) than in case of salt stressed sunflower seedlings (fig.3). Proline can be used as a metabolic marker in relation to stress. Proline produces immediately after encounter of cells with salt stress and protects the plasma membrane and proteins against stress (Santoro et al. 1992).

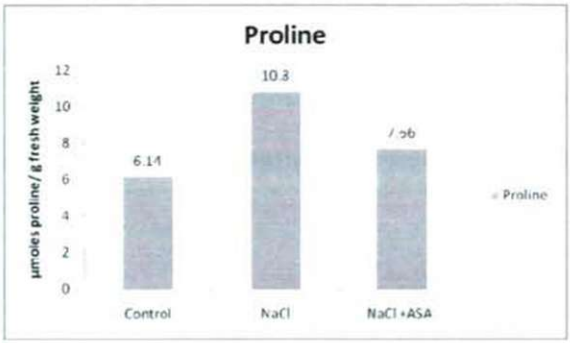

Figure.3 Proline content in sunflower seedling in stressed or unstressed condition with or without ASA treatment

Studying the influence of ASA on the isolates pathogen, we observed significant differences in the colony diameter depending on the used concentration. The 20mM concentration both in crystal and powder form of ASA assured significant limitation in the grows of *Botrytis cinerea* and *Sclerotinia sclerotiorum* colonies after 24 and 96 hours (table 4 and 5).

Studies regarding the influence of AS on seed germination shows a positive effect of ASA powder applied in sunflower seed dressing on the percentage of seed germination and frequency of infected plants, after a 7 day incubation period (table 6).

Table 4. Influence "in vitro" of ASA incorporated in PDA the grows of Botrytis cinerea isolated from sunflower -colony diameter in cm.

Concentration	ASA applied			
	Crystals		Powder	
	after 24 hours	after 96 hours	after 24 hours	after 96 hours
2,5 mM	0.20 $^{++}$	7.00	0.90	2.00
5 mM	0.20 $^{++}$	7.00	0.65 ^{o}	1.25 ^{ooo}
10mM	0.20 $^{++}$	7.00	0.45 ^{oo}	1.10 ^{ooo}
20mM	0.10 ^{oo}	6.00 ^{oo}	0.40 ^{oo}	1.00 ^{ooo}
Check	0.15	7.00	1.00	2.00
SD 5%	0.03	0.09	0.31	0.24
SD 1%	0.04	0.14	0.43	0.33
SD 0,1%	0.06	0.19	0.61	0.47

Table 5. Influence "in vitro" of ASA incorporated in PDA on growing of Sclerotinia sclerotiorum isolated from sunflower -colony diameter in cm.

Concentration	ASA applied			
	Crystals		Powder	
	after 24 hours	after 96 hours	after 24 hours	after 96 hours
2,5 mM	0.700	7.000	0.250 ^{ooo}	2.000 ^{ooo}
5 mM	0.700	7.000	0.325 ^{ooo}	0.650 ^{ooo}
10mM	0.700	7.000	0.125 ^{ooo}	0.525 ^{ooo}
20mM	0.700	5.275 ^{ooo}	0.000 ^{ooo}	0.250 ^{ooo}
Check	0.700	7.000	2.000	3.000
SD 5%	-	0.25	0.22	0.24
SD 1%	-	0.35	0.31	0.34
SD 0,1%	-	0.49	0.44	0.48

Table 6. Influence of ASA applied in seed dressing on seed germination and frecquency of Bortytis cinerea and Sclerotinia sclerotiorum infection in sunflower

Seed treatment	Dose g/kg	% seed germination	F% infected plats	
			S. sclerotiorum	B. cinerea
Metoben 70WP	2	95.5	1.0	2.0
Rovral 50WP	2	95.0	1.0	1.5
ASA powder	1	93.5	2.0	3.5
Check (untreated)	-	89.5	3.5	8.5

- 7 day incubation in humid chamber

Studying the influence of ASA applied in seed dressing performed in the experimental plots of ARS Oradea, shows the positive influence on seed emergency percentage in field conditions, in comparison with the untreated check and two fungicides (Metoben 70 WP and Rovral 50 WP) usually used in seed dressing of sunflower. There was applied artificial inoculation at the sowing using mycelium and sclerotia of the fungus grows on autoclaved barley seed. Our results indicated also a limitative effect on the frequency of infected plants in comparison with the untreated check. The yield difference registered in these conditions (150 kg/ha, respective 10.3%) after the seed treatment with ASA powder (1 g/kg seed) shows the positive but not significant effect of ASA in the assured high infection pressure (table 7) and the necessity of the extension of our research in this direction.

Table 7. Influence of ASA applied in seed dressing on frecquency of Sclerotinia sclerotiorum infection and yield level in sunflower- artificial inoculation with mycelium and sclerotia grows on autoclawed barley seed.

Seed treatment	Dose g/kg	% of emergence	F% infected plats S. sclerotiorum	Yield kg/ha	Differences	
					kg/ha	%
Metoben 70WP	2	95.5	1.0	1750	+300++	20.7
Rovral 50WP	2	95.0	1.0	1700	+250++	17.2
ASA powder	1	93.5	2.0	1600	+150	10.3
Check (untreated)	-	89.5	3.5	1450	-	-

SD 5% 180 kg/ha
SD 1% 245 kg/ha
SD 0.1% 350kg/ha

The repeated field test in condition of natural infection confirms the favorable effect of seed dressing on the plant emergency percentage, on the frequency of natural infected plants and also on the yield quantity. The yield difference registered in comparison with the untreated check in this case was significant (table 8). These promising positive results motivates the necessity of the extension of our research in this direction.

Table 8. Influence of ASA applied in seed dressing on frecquency of Sclerotinia sclerotiorum infection and yield level in sunflower.

Seed treatment	Dose g/kg	% of emergence	F% infected plats S. sclerotiorum	Yield kg/ha	Differences	
					kg/ha	%
Metoben 70WP	2	95.7	0.5	2150	+200++	10.2
Rovral 50WP	2	96.5	0.5	2270	+220++	11.3
AAS powder	1	94.5	0.7	2100	+150++	7.7
Check (untreated)	-	90.5	1.9	1950	-	-

SD 5% 105 kg/ha
SD 1% 145 kg/ha
SD 0.1% 225 kg/ha

4. CONCLUSION

- The results obtained after pre-treatment of sunflower seeds with ASA may cause a low level of oxidative stress, improving the antioxidative capacity of the plants, increasing the plant tolerance to salt stress induced in our experiment by 150 mM NaCl treatments and the total clorophyllian pigment content under salt stress.
- The presence of ASA in PDA nutrient medium assured significant limitative effect in the colony diameter of two pathogenic fungi (*Botrytis cinerea* and *Sclerotinia sclerotiorum*), this effect depending on the used concentration.
- ASA applied in sunflower seed dressing performed in the field plots of ARS Oradea, shows the positive influence on seed emergency percentage, limiting the level of infected plants with *Sclerotinia sclerotiorum* in field conditions, in comparison with the untreated check and two large used fungicides. In condition of natural infection seed treatment had significant effect also on the yield level.

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